

Modified Recombinant Allergens for Safer Immunotherapy

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Abstract: Molecular cloning and recombinant production of allergens offered new perspectives for the increasing problem of allergies. A variety of preparations are being developed aiming to increase safety and improve efficacy of specific immunotherapy. Recombinant-based approaches are mostly focused on genetic modification of allergens to produce molecules with reduced allergenic activity and conserved antigenicity, i.e. hypoallergens. Studies dealing with genetic modifications of allergen genes reported the production of site-directed mutants, deletion mutants, allergen fragments and oligomers, and allergen chimeras. An alternative to genetic engineering is the chemical modification of pure recombinant allergens. It has been shown that allergens modified with immunostimulatory DNA sequences (allergen-ISS conjugates), which masks IgE epitopes and adds a desirable Th1-inducing character to the allergen molecule. Other chemical modifications include oligomerization by aldehydes (allergoids) and maleylation, which seems to target allergens to particular antigen presenting cells. Several of these modified allergen preparations have been already evaluated for their safety in clinical provocation studies. So far, clinical trials showed the efficacy and safety of immunotherapy with an Amb a 1-ISS conjugate for ragweed pollen-allergic patients. In addition, a preparation consisting of hypoallergenic fragments of Bet v 1 was evaluated for immunotherapy of birch pollen-allergic patients. In parallel, several animal studies have now demonstrated the potential of genetic immunization for allergy treatment in the future.

Keywords: Recombinant allergen, IgE epitope, T cell epitope, allergen vaccines, specific immunotherapy, hypoallergen, genetic engineering, DNA vaccines.

1. INTRODUCTION

Allergen-Specific Immunotherapy (SIT): From Whole Extracts to Engineered Allergen Vaccines

The most effective allergy treatment would be the one that specifically and with a maximum of safety cures clinical symptoms caused by allergens in any given patient. In this context, SIT offers the best possibility of accomplishing this aim. The process is highly specific as the treatment is targeted at those allergens, which the patient and the physician identified as the responsible ones [1]. In its conventional form, SIT involves repeated administration of whole allergen extracts to patients displaying IgE-mediated allergic symptoms and inflammatory reactions caused by natural exposure to allergens. It has been used since 1911 and represents the only curative approach for type I allergy. In addition, SIT is capable of modifying the natural course by preventing the worsening of symptoms (from rhinitis to asthma) and the onset of sensitization against new allergens [2-4].

Although this type of therapy is widely established, four major problems are still associated with SIT: (i) SIT is performed with allergen extracts (containing mixtures of allergens, non-allergenic and/or toxic proteins, and other macromolecules) that are difficult to standardize; (ii) induction of new IgE specificities has been reported for birch and grass pollen allergic individuals [5, 6]. 65% of patients treated with birch pollen extract rush immunotherapy developed low levels of IgE antibodies towards additional

birch pollen allergens; (iii) severe IgE-mediated side effects can occur during the treatment due to systemic administration of fully active allergens; (iv) therapeutic effective doses often cannot be achieved because of non-standardized extracts or side effects.

These problems could in principle be solved by the use of recombinant allergens (reviewed in [7-12]). Cocktails of pure and standardized recombinant allergens can be formulated to substitute natural extracts and the selected recombinant allergens can be modified to reduce the risk of IgE-mediated side effects.

2. MODIFYING RECOMBINANT ALLERGENS TO IMPROVE SIT

Modification of allergens to improve conventional SIT should aim at the production of molecules with reduced IgE binding epitopes (hypoallergens), while preserving structural motifs necessary for T cell recognition (T cell epitopes) and for induction of IgG antibodies reactive with the natural allergen (blocking antibodies). The uptake of allergens by antigen-presenting cells (APCs) is mediated and facilitated by the interaction of the allergen with specific IgE [13, 14] and leads to higher Th2 cytokines and IgE production [15]. Modified allergens lacking IgE binding (hypoallergens) might avoid these pathways and preferentially target APCs that utilize phagocytosis or pinocytosis for antigen uptake (e.g. monocytes, macrophages, and dendritic cells). This in turn induces a balanced Th0 or Th1-like cytokine production by T cells, low IgE and high IgG production by B cells. Akdis and co-workers [15] showed that bee venom phospholipase A2 (PLA) preparations lacking the native conformation and antibody-binding activity were exclusively presented by monocytes and induced a Th1-biased cytokine

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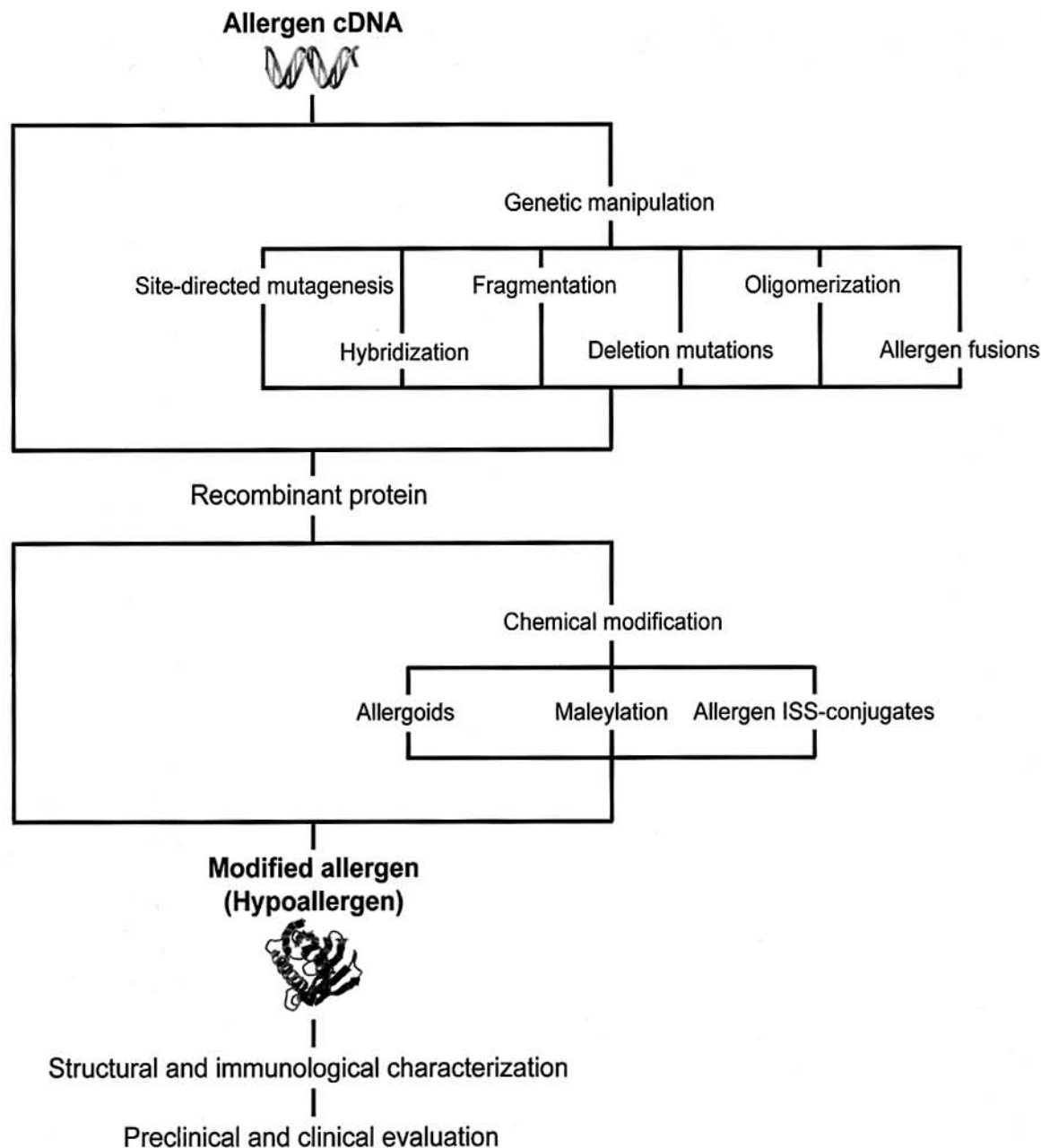


Fig. (1). Task tree and approaches for modifying recombinant allergens for specific immunotherapy. Although not yet explored, chemical modification of genetically engineered allergens could be an interesting new procedure for producing hypoallergenic vaccines. Candidate molecules should be subjected to intensive characterization before evaluation in clinical trials.

profile leading to IgG4 production by B cells. In contrast, folded PLA with full antibody-binding activity was processed and presented by B cells, stimulated Th2-like cytokines and induced IgE antibodies. Thus, the three-dimensional structure of an antigen and its recognition by different APCs seem to be crucial aspects in the development of distinct patterns of T cell cytokines.

As mentioned above, the presence of intact T cell epitopes on hypoallergens would target T cells allowing administration of higher doses to induce tolerance of allergen-specific T cells and alteration of cytokine production towards a Th1-like pattern. In this way, vaccine preparations consisting of hypoallergens could replace natural extracts and increase the efficacy and safety of SIT.

As discussed below, three major groups of hypoallergens can be presently distinguished: chemically modified allergens, naturally occurring hypoallergens, and genetically modified allergens. Approaches used to modify recombinant allergens for SIT are outlined in Fig. 1.

2.1. Chemically Modified Allergens

The idea of modifying allergens in order to decrease their allergenicity (IgE-binding activity) while preserving their antigenicity (T cell reactivity) is not new. Earlier studies showed that chemical modification of allergens (e.g. polyethylene glycol, glutaraldehyde, formaldehyde, etc.) could be used to abrogate IgE binding. Some of these

preparations (allergoids) are currently available in the clinical practice [9]. The experience with these chemically modified preparations encouraged the idea of designing hypoallergenic vaccines based on recombinant proteins. One advantage is that chemical modifications can be applied to almost every allergen molecule, as they are based on chemical properties of proteins in general.

Oligomeric and Monomeric Allergoids

Oligomerisation produced by chemical modification of allergens with glutaraldehyde or paraformaldehyde (allergoids) seems either to destroy or to mask structural B-cell epitopes resulting in significantly reduced IgE binding activity while maintaining immunogenicity. Allergoids seem to be better tolerated by patients, higher doses of antigen can be administered during SIT and consequently fewer injections are necessary for a successful treatment [9, 16].

Chemically modified allergens with reduced allergenic potential can also be produced by carbamylation. Treatment of proteins with potassium cyanate at neutral pH leads to a transformation of the ϵ -amino group of lysines into ureido groups. In this way, the native monomeric character of the allergens is preserved. One advantage is that monomeric allergoids can be administered to the organism not only subcutaneously but also through a mucosal pathway. In animal models, monomeric allergoids induced IgG antibodies that recognized the native allergens [17].

Presently, commercially available allergoids are produced by chemical modification of allergen extracts with aldehydes, and are only available for a limited panel of allergens. They consist of bulky macromolecules with undefined structure and sequence. It would seem a better approach for the production of allergoids the use of recombinant allergens instead of allergen extracts. In this respect, allergoids prepared from rPhl p 5b induced a somewhat lower T cell proliferative response of Phl p 5-specific TCC using PBMC as APC, when compared with purified rPhl p 5b or untreated extract. rPhl p 5b allergoid was efficiently presented by macrophages and dendritic cells but not by B cells, whereas rPhl p 5b was presented by all APCs, including B cells [18]. This is an indirect evidence that allergoids bypass Ab-mediated allergen uptake and are targeted to APCs using fagocytic or pinocytic uptake mechanisms.

Maleylated Allergens

An interesting approach explores the observation that maleylation (alteration of the ϵ amino group of lysine residues by treatment with maleic acid anhydride) of protein antigens converts them into SR ligands and their delivery through SRs results in increased immunogenicity with a dominant Th1-type immune response [19, 20]. SRs are members of the so-called pattern recognition receptors, which are expressed by antigen presenting cells and are involved in the recognition of foreign ligands during early phases of the immune response [21]. Thus, specific targeting of allergens to SR could be an attractive approach to modulate allergen-specific immune responses. Maleylation of whole Orchard grass pollen proteins was reported to decrease IgE binding activity tested by immunoblots and to reduce skin reactivity [22]. A maleylated derivative of shrimp tropomyosin, a major cross-reacting crustacean food

allergen, displayed reduced allergenicity as compared to the native counterpart [23]. Analysis of the cytokine profiles after stimulation of splenocytes isolated from Balb/c mice immunized with maleylated tropomyosin showed a dominant Th1 immune response. Similar results were obtained by targeting a Der p 1 peptide to macrophages via SR [24]. In addition, SR targeting of the Der p 1 peptide and elicitation of Th1-dominant responses was also accomplished by simple co-adsorption of the peptide with maleylated poly-L-lysine on alum [24]. The possibility of co-adsorption could find applications in the formulation of multi-vaccines for immunotherapy of patients allergic to several allergens.

Although there are no reports specifically addressing this topic, maleylation of recombinant allergens or allergen-derived non-anaphylactic peptides could be an attractive approach for producing and specifically targeting hypoallergens.

Allergen-ISS Conjugates (AIC)

Recent developments for chemical modification of allergens include conjugation with synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG sequences from bacterial DNA. CpG-ODN conjugated to allergen can mask allergenic determinants thus interfering with cross-linking of Fc IgE receptors on mast cells and basophils [25]. Direct coupling of allergens to CpG-ODN was shown to be superior to a mixture of CpG-ODN and allergen with respect to immunogenicity. An explanation for this would be that both the allergen and the CpG motifs are delivered together to the same APC for processing and induction of type 1 cytokines secretion. Indeed, much less CpG-ODN in the form of an AIC was necessary to induce the same IgG2a antibody titer as compared to allergen CpG-ODN mixtures. AIC induced a Th1 response to the allergen as shown by strong IgG2a and almost no IgE anti-allergen antibody production and antigen specific IFN- γ secreting spleen cells. This is in contrast to control mice, which produced IgG1 and IgE antibodies and showed elevated levels of IL-5. Sensitization with allergen and treatment with AIC induced IgG2a antibodies without significantly increasing the IgE titer and spleen cells from AIC treated mice showed significantly more IFN- γ secretion than spleen cells from the control groups, both pointing to a therapeutic potential of AICs [26].

An AIC with an average of four CpG-ODN conjugated per molecule of Amb a 1 was tested for safety, tolerability and immune response in comparison to a licensed ragweed allergen extract in a phase I trial [27]. This clinical study with a quantitative intradermal procedure indicated that more than 100-fold higher quantities of Amb a 1 in the form of AIC were necessary to induce the same wheal and flare skin test reaction as native ragweed allergen. Preliminary data from early phase I/II clinical trials showed that the Amb a 1-specific IgE response was similar among study subjects who received subcutaneous injections of AIC and those who received placebo injections. In contrast, IgG anti-Amb a 1 antibody titers after six weekly injections with AIC appeared comparable to titers measured in sera from individuals who received immunotherapy for more than one year with licensed ragweed allergen extracts [28, 29]. Thus, AIC seem to display the required features of modern vaccines for an

improved SIT such as a high safety profile and strong immunogenicity. In addition, the curative principle can be clearly attributed to the balancing of the allergic Th2-type response by counteracting with Th1-inducing stimuli. Although the Amb a 1-AIC was produced using purified natural Amb a 1, recombinant-based AIC preparations would be the obvious development of AICs for SIT.

2.2. Naturally Occurring Hypoallergens

cDNA cloning showed that several major allergens are encoded by gene families. Due to sequence variations, isoallergens might display different antigenic and/or allergenic activities. Differences in T cell reactivity of isoforms have been reported for Cor a 1 [30], Bet v 1 [31], Phl p 5 [32, 33], and for Der p 2 [34].

Dissection of the immunological properties of isoallergens led to the identification of naturally occurring hypoallergens of Bet v 1, the major birch pollen allergen [31]. Recombinant isoforms Bet v 1d, Bet v 1g and Bet v 1l were found to be highly antigenic in T cell proliferation assays and low in their allergenic activities *in vitro* [31] and *in vivo* by skin testing [35]. The crystal structure of the hypoallergenic isoform Bet v 1l in its recombinant form was recently determined [36] and shown not to be significantly different from the structure of the high IgE binding isoform Bet v 1a [37]. Such well-characterized molecules would be excellent candidates for specific immunotherapy. Unfortunately, naturally occurring hypoallergens have not been identified for other allergen families. Instead, genetic engineering has been widely used to generate hypoallergens.

2.3. Genetically Modified Allergens

Genetic engineering involves the targeted modification of a protein in order to alter its function or properties in a predictable manner. Therefore, understanding the relationship between the structure of the encoded protein and its function/properties is a pre-requisite for the precise and effective manipulation of a gene. Approaches for the alteration of a gene include changing specific base-pairs (mutated gene), introduction of a new piece of DNA into the existing DNA molecule (chimeric or hybrid gene, head-to-tail fusions), and deletions (truncated gene or fragments). So far, cDNA sequences of approximately 400 allergen genes have been deposited in the allergen databank (www.allergen.org). Thus the starting material for producing new vaccines for important allergies is available. Modification of allergen genes usually requires knowledge of B and T cell epitopes and in some cases also of the three-dimensional structure of the native allergen. An exception is the DNA shuffling procedure, which bypasses the need to identify critical amino acid residues or motifs. But independent of how allergen genes are altered, putative hypoallergens must be subjected to a series of *in vitro* and *in vivo* evaluation procedures, before being considered for therapeutic purposes (Fig. 1).

Site-Directed Mutants

Data concerning crucial amino acid residues or motifs involved in IgE recognition of allergens can be used to modify dominant B-cell epitopes by site-directed

mutagenesis. We analyzed the patterns of amino acid substitutions in Bet v 1 isoallergens and homologues and their IgE-binding activities using a computer algorithm developed to predict functional residues in protein sequences. Based on this analysis, a Bet v 1 mutant carrying six point mutations was produced displaying low IgE-binding activity for all patients tested [38]. *In vivo* (skin prick) tests indicated that the potency of the six-point mutant to induce typical urticarial skin reactions in allergic individuals was dramatically reduced (100- to 1000-fold) compared to Bet v 1a, a hyperallergenic isoform. In addition, Bet v 1a-specific T cell clones (TCC) established from the peripheral blood of birch pollen-allergic patients proliferated in response to the six-point mutant. In another study, a dominant IgE-binding epitope of Bet v 1 was identified by X-ray crystallography and site-directed mutagenesis [39].

IgE recognition of polcalcins, a group of calcium-binding allergens found in various pollen [40], and parvalbumin, a cross-reactive fish allergen [41], is influenced by bound calcium. This finding led to the idea of disrupting the EF-hand calcium-binding domains for engineering hypoallergenic mutants. Calcium-binding deficient mutants of Bet v 4 [42] and Bra r 1 [43] with reduced IgE-binding activities were generated. Information concerning T cell recognition of calcium-binding allergens and their engineered counterparts is not available.

The introduction of point mutations in highly conserved sequence domains of Lol p 5 was based on investigations of B- and T- cell epitopes and sequence comparison of group 5 allergens from different grasses. Hypoallergenic forms of Lol p 5 were produced containing all relevant T cell epitopes [44]. Likewise, hypoallergenic variants of latex hevein Hev b 6.02 [45] and Hev b 5 [46], apple Mal d 1 [47], egg Gal d 1 [48], and peanut Ara h 1, Ara h 2, and Ara h 3 [49] were also generated by site-directed mutagenesis. Modified peanut allergens retained their ability to stimulate T cell proliferation in PBMC from allergic patients. In addition, peanut hypoallergens have been tested in a murine model of peanut anaphylaxis [50] and shown to be effective.

Hypoallergenic variants of the major allergen of Par j 1 displaying altered conformation were constructed [51]. Par j 1 is a member of the non-specific lipid transfer proteins (nsLTPs) with a characteristic α - α - α - α - β structure that is stabilized by four disulfide bonds [52]. Targeting these disulfide bonds by site-directed mutagenesis resulted in molecules with altered conformation and decreased IgE-binding activity and conserved T cell reactivity. Recently, the Par j 1 hypoallergens were also evaluated *in vivo* in a murine model of allergic sensitization [53]. Disruption of the native conformation by targeting disulfide bonds could be an approach generally used for engineering hypoallergenic nsLTPs, including food-derived members. Disulfide bonds stabilizing the antigenic structure of major allergens of house dust mites were also targeted by site-directed mutagenesis. Hypoallergenic variants of Der p 2 [54], Der f 2 [55], and Lep d 2 [56, 57] were produced and evaluated for their IgE-mediated reactions and cellular responses. One potential problem when targeting the conformation of allergens could be the solubility of the final product, as denatured or unfolded proteins tend to form aggregates. Protein

preparations consisting of aggregates or insoluble molecules would not be suitable candidates for therapeutic products.

Deletion Mutants

Deletion of DNA segments in the gene corresponding to IgE-binding epitopes is an interesting possibility for modifying allergen genes. This approach was successfully used for the timothy grass pollen allergen Phl p 5b [58]. Epitope mapping was performed using overlapping recombinant fragments and at least 4 continuous IgE-binding epitopes were identified. Deletions avoiding identified T cell epitopes were then performed within these IgE-binding regions. Some of these deletion mutants showed reduced IgE-binding properties, no histamine-releasing activity, reduced skin reactivity, and no significant changes in T-cell reactivity. A similar approach was used for the American cockroach *Per a 1* allergen [59].

Based on the results obtained by proteolytic fingerprinting, a deletion mutant of the ryegrass Lol p 1 allergen was produced displaying decreased IgE-binding activity. The Lol p 1 mutant did not trigger histamine release up to a concentration of 10 mg/ml [60].

Allergen Fragments

Disruption of the three-dimensional structure by fragmentation could be used to reduce the anaphylactic potential of allergens due to destruction of major IgE-binding epitopes. IgE epitopes can be formed by a stretch of amino acids in a row (continuous epitopes) or by non-adjacent sequence elements brought together by folding (discontinuous or conformation-dependent epitopes). IgE recognition of continuous epitopes might also depend on their conformation, which might only occur in the context of the folded allergen molecule. Following this line, non-anaphylactic fragments of the major house dust mite allergen Der f 2 were produced by C- and N-terminal deletions, and were mixed after separate refolding of the denatured fragments [61]. Fragments of the calcium-binding allergens Bet v 4 [62] and Aln g 4 [63], and an N-terminal fragment of Lol p 1 from ryegrass [60] also showed decreased IgE binding activities. However, they were not tested for T cell reactivity and immunogenicity.

The three-dimensional structure of Bet v 1 was disrupted by expressing in *E. coli* two fragments of the cDNA corresponding to amino acids 1-74 and 75-160 [64]. The fragments exhibited random coil conformation and almost no allergenicity. Together, the fragments harbored all relevant T cell epitopes. Skin reactivity and histamine release were greatly reduced when compared to the native intact Bet v 1 allergen [65, 66]. Further, immunization of mice and rabbits with Bet v 1 fragments induced IgG antibodies that inhibited binding of IgE from allergic patients to wild-type Bet v 1 [67].

Recently, the Bet v 1 fragments were used to vaccinate 124 birch pollen-allergic patients in a double-blind, placebo-controlled study [68]. Active treatment induced protective IgG antibodies that inhibited allergen-induced release of inflammatory mediators. The authors also observed a reduction of cutaneous sensitivity and an improvement of symptoms in actively treated patients. In addition, increases of allergen-specific IgE induced by seasonal birch pollen

exposure were significantly reduced in vaccinated patients. This was the first clinical trial using a modified recombinant allergen preparation.

Allergen Oligomers

Oligomeric forms of the major birch pollen allergen Bet v 1 were also constructed [69]. Two or three copies of the full-length Bet v 1 molecule were linked by short spacers, expressed in *E. coli* and purified to homogeneity. *In vitro* studies using patients sera showed comparable IgE binding for the rBet v 1 monomer, dimer and trimer whereas the trimer showed reduced histamine release of patients' basophils compared to the monomer. This reduced anaphylactic potential was verified *in vivo* by skin testing of birch pollen allergic patients. When compared to the Bet v 1 monomer, the Bet v 1 trimer induced similar proliferation and cytokine production upon stimulation of Bet v 1-specific T cell clones. *In vivo* studies in animal models showed the ability of the Bet v 1 trimer to induce IgG blocking antibodies, which inhibited binding of human IgEs to rBet v 1 [65, 66, 70].

Chimeras: Allergen Hybrids and Shuffled Molecules

King and co-workers [71] reported an approach to modify allergens in which hybrids are prepared consisting of a small portion of the guest allergen of interest and a large portion of a homologous but weakly cross-reacting host protein. The idea is that the homologous host protein serves as a scaffold to maintain the native structure of the guest allergen of interest in order to preserve conformational-dependent B cell epitopes, but at a reduced density. The homologous allergens from yellow jacket venom Ves v 5 and from paper wasp Pol a 5 (59% sequence identity) show very limited cross-reactivity of antibodies from sensitized patients. Hybrids of these two molecules containing 10-49 residues of Ves v 5 showed 100- to 3000-fold reduction in allergenicity determined by histamine release assays with basophils from yellow jacket-sensitized patients. Such approach could be employed for several allergens with known homologues displaying low allergenic activity. However, it still remains to be established if injections of such hybrids would induce novel IgE specificities.

Another promising approach to generate allergen chimeras is the DNA shuffling, which consists in the fragmentation of two or more related genes and their random re-assembly followed by an amplification step. Because of sequence similarities of the parental genes, the gene fragments can form areas of recombination during the "shuffling" process resulting in the generation of new full-length genes. Libraries created in this way consist of highly diverse sequences and can be subjected to a diversity of screening procedures for the selection of molecules fulfilling the desired requirements [72, 73].

DNA shuffling seems as an ideal strategy to be exploited for generating novel allergy therapeutics. Diminished IgE reactivity could either be achieved by minimizing the number of epitopes per shuffled molecule to zero or one, which would avoid cross-linking of IgE receptors on effector cells. Further the affinity of IgE antibodies toward these epitopes could be reduced. Nevertheless, the shuffled products should be able to influence the T cell population of an allergic individual by either shifting the typical Th2

towards a Th1 response or by inducing T cell tolerance. Considering cross-reactive allergens, the generation of allergen chimeras associating T cell epitopes from several allergens but at the same time with extremely reduced anaphylactic potential also becomes possible by applying DNA family shuffling. Chimeric proteins obtained by family shuffling could not only be beneficial for allergy treatment but also for diagnostics, especially when considering allergens with higher IgE binding activities [74-76].

In our preliminary experiments, a group of pollen allergens derived from birch, hazel and alder pollen, all belonging to the highly cross-reactive Bet v 1 family, were selected for DNA family shuffling. The resulting chimeric allergens were shown to be composed of several epitopes derived from different parental genes. Interesting results were obtained regarding the IgE reactivity of the chimeric Bet v 1 molecules when tested with birch pollen allergic patients' sera. In these experiments, candidate molecules showing either elevated or diminished IgE binding activity compared to the parental proteins could be identified. The antigenicity of the chimeric Bet v 1 molecules was comparable to wild type Bet v 1a, as determined by *in vitro* T cell proliferation assays using T cell lines from Bet v 1-sensitized mice and Bet v 1-specific T cell lines derived from birch pollen allergic individuals [76].

Allergen Fusions

An alternative approach consisting of a fusion of two unrelated allergens from the same species was implemented in a study with the timothy grass pollen allergens Phl p 1, Phl p 2, Phl p 5 and Phl p 6, respectively [77]. Dimeric fusion molecules (rP2-P6, rP6-P2 and rP5-P1) were constructed, which showed similar secondary structures at equimolar mixes of the individual allergens, as determined by far-UV CD. IgE inhibition experiments revealed that the fusions represented the full spectrum of B-cell epitopes and all three fusions induced strong proliferative response in PBMCs from grass pollen allergic patients. It was also observed that the immunogenicity of these hybrids was altered compared to the parental allergens. Molecules showing low immunogenicity as monomers displayed increased potential of T-cell stimulation as fusion partners. These findings offer novel possibilities in terms of specific immunotherapy. Since allergen extracts used for immunotherapy bear not only the risk of de novo sensitization towards new allergens, also the different allergens administered are not equally efficient in stimulating the human immune system. The latter seems also to be true for equimolar combinations of recombinant allergens. By fusing grass pollen allergens, it was possible to increase the immunogenicity of allergens displaying weak immunogenic activity. Ideally, this approach should fuse modified versions of the allergens displaying low IgE-binding activity. In this way, safer combinatorial allergy vaccines could be produced.

3. DNA-BASED VACCINES TRANSLATING MODIFIED ALLERGEN GENES

Injection of plasmid DNA encoding clinically relevant allergens can induce long-lasting immune responses with a Th1 bias and promote the formation of IFN- γ -producing CD4⁺ T cells. These IFN- γ -secreting Th1

cells can stimulate B cells to synthesize IgG2a antibodies and inhibit IgE production in mice [78-82]. Therefore, genetic immunization is an attractive alternative for immunotherapy using allergenic proteins. However, it has been shown that after subcutaneous administration of plasmid DNA encoding an allergen, transcripts were simultaneously detected in several tissues [80]. This raises the concern that the anti-allergic Th1-inducing delivery mode of plasmid DNA based on needle injection would result in a number of cells expressing the allergen in different tissues sufficient to trigger IgE-mediated side effects. Therefore it would seem advantageous to develop DNA-based vaccines encoding hypoallergenic variants (e.g. site-directed mutants, fragments, chimeras, etc) instead of using wild-type genes.

To evaluate this concept, DNA vaccines coding for two hypoallergenic Bet v 1 fragments [67] as well as for a genetically engineered Bet v 1 hypoallergen [38] were constructed. Immunization with both types of constructs protected in an antigen-specific manner from IgE production and IgE-mediated cell release after repeated sensitization with recombinant allergen [83]. Compared to the wild-type DNA vaccine that stimulates a strong humoral response, the DNA vaccine encoding the hypoallergenic mutant of Bet v 1 triggered a reduced and transient antibody response against wild-type Bet v 1, thus indicating the altered allergenic and immunogenic properties of the modified allergen. The vaccine encoding the two hypoallergenic fragments of Bet v 1 stimulated no antibody response against Bet v 1 confirming that the fragments do not display allergy-associated IgE epitopes present on the full-length wild-type Bet v 1 molecule. Further, the fragment vaccine triggered the recruitment of Bet v 1-specific Th1 cells as indicated by IgG2a antibodies, IgE suppression and antigen-specific IFN- γ production after sensitization with the native allergen. A therapeutic approach with sensitization followed by DNA immunization with the fragment-DNA vaccines revealed a remarkable and sustained antigen-specific reduction of IgE antibodies and IgE-mediated release [83] and confirmed the excellent therapeutic potential of this type of DNA-based vaccine.

4. SHORT OVERVIEW OF ALTERNATIVE TREATMENTS AND ADJUVANTS SUPPORTING SIT

4.1. Anti-IgE Therapies

Several strategies were devised to treat IgE-mediated allergic diseases by down-regulating IgE levels [84-87]. The general concept was based on chimerized or humanized anti-IgE antibodies with a set of unique binding properties, which could be used for the isotype-specific control of IgE, and thus would be a general therapeutic approach to IgE-mediated diseases. An anti-IgE antibody must fulfill several criteria: the anti-IgE antibody must have a high affinity for IgE, should not bind IgE already bound by Fc ϵ RI on mast cells and basophils, nor to IgE bound by the low-affinity IgE Fc receptors (Fc ϵ RII, also known as CD23) on various other cell types and should bind to membrane-bound IgE (mIgE) on mIgE-expressing B cells. Summarizing, these antibodies are designed to neutralize free IgE and target IgE-expressing B cells [84, 86, 88]. If these aims are achieved, the levels of

IgE in blood and interstitial fluids for binding to FcεRI will be greatly reduced, and hence the sensitivity of mast cells and basophils to allergens should be gradually alleviated. On the other hand, as the anti-IgEs do not bind to IgE bound by FcεRI, they do not crosslink FcεRI-bound IgE and sensitize mast cells and basophils, in contrast to anti-IgE antibodies that do not possess these binding properties [89]. Also, as the anti-IgEs do not bind to IgE bound by FcεRI—which is expressed broadly on lymphocytes, macrophages, platelets, and many other cell types—they are not expected to cause any adverse effects associated with such binding. The anti-IgEs under clinical evaluation and development have an association constant, K_a , for soluble IgE of approximately 10^{10} M⁻¹ [90], about the same as that of FcεRI for IgE. Thus, if anti-IgE is maintained at concentrations in excess of IgE in the body, it should effectively compete with FcεRI for IgE. The concentrations of IgE in the blood vary widely among patients with allergy, ranging from 0.05 to 1 mg/ml in most patients. Taking this into account, anti-IgE given in excess to the basal concentration can bind most of IgE, leaving a minimum of free IgE to bind to IgE-Fc receptors.

Effects on IgE Receptors and IgE Producing B Cells

Probably the most readily appreciated pharmacological effect of anti-IgE therapy is its indirect effect on the downregulation of FcεRI on basophils. In earlier studies, the density of FcεRI on basophils was found to correlate strongly with the level of IgE in blood. In one of the clinical studies [91] and in additional *in vitro* studies [92], it was found that anti-IgE can also downregulate FcεRI in patients. The results show that the density of FcεRI on basophils had decreased by more than 95%, and in some cases to more than 99%, after anti-IgE was administered to patients for periods of five weeks to three months [91]. The basophils isolated from patients after anti-IgE treatment were much less sensitive to allergen stimulation. In skin prick tests, it was also found that much larger amounts of allergens were required, indicating that mast cell function was also markedly decreased [91].

Experimental evidence from *in vitro* and *in vivo* studies is generally supportive of the effectiveness of anti-IgE in targeting IgE expressing B cells and in inhibiting the continual production of IgE. The explanation for these effects is that anti-IgE binds to mIgE on IgE-expressing B cells, and as mIgE is a part of the B-cell receptor, anti-IgE may inhibit the B cells or even cause their lysis, like anti-IgM or anti-IgG [93-95]. However, IgE-secreting plasma cells do not express mIgE and presumably are not affected by anti-IgE. These cells reside in the bone marrow and probably have a life span of several weeks to several months. Since new IgE-secreting plasma cells go through mIgE-expressing B cell stages during differentiation, if their generation is abrogated by anti-IgE treatment, the existing plasma cells will die off in several weeks to several months, and thus the production of IgE will also gradually abate in similar periods. Furthermore, memory B cells may possibly be affected by anti-IgE. If this occurs, anti-IgE may have long-term effects on the fundamental disease process. The molecular mechanisms, leading to the depletion of these cells can be explained by apoptosis and reached by the immunological process of tolerance and/or anergy induction. Transgenic animal studies confirmed that clonal deletion and

clonal anergy are the principle mechanisms of self-tolerance. Clonal deletion is the physical elimination (death) of a B cell, while clonal anergy is the silencing of a B cell so that while it is still alive, it no longer produces antibody, nor can it be readily activated [96]. Both mechanisms and / or strategies naturally occur during B cell development. Transferring these ideas to an affinity matured, class-switched IgE B cell population, two scenarios of the functioning of an anti IgE antibody would be possible: First, as shown by self reacting immature B cells, cross linkage of the mIgE receptor without further T cell support should directly induce apoptosis. Second, as normally shown during the induction of peripheral tolerance of mature B cells in answer to monovalent (self) antigen, receptor blockage of mIgE by a Fab-fragment should result in an anergic state of the mIgE population. In contrast to normal mature B cells, which have a half-life of 4 to 5 weeks, anergic B cells were found to last for only 3 to 4 days [97]. Nevertheless the fate of these anergic B cells finally turns out to be cell death [98].

Summarizing clinical applications and studies so far, the most advanced therapeutic approach for the treatment of allergic diseases is the treatment with recombinant humanized anti-IgE antibodies. Targeting IgE is a systemic approach against an increased IgE production.

Anti-IgE and SIT

Patients suffering from allergic rhinitis are often sensitized to multiple outdoor allergens. For these patients SIT is the only available approach so far. Despite optimized treatment regimens, including antihistamines, corticosteroids and mast cell stabilizers, a subgroup of patients with allergic rhinitis have insufficient symptom control [99]. Anti-IgE therapy was shown to be effective not only for seasonal allergic rhinitis, but has also been successfully tested for allergic bronchial asthma and food allergy. The optimal duration of anti-IgE therapy is unknown, but it is likely that anti-IgE has to be administered continuously in a dose dependent fashion. Furthermore, anti-IgE therapy seems to be effective in all age groups that have been studied so far. Therefore, new treatment options, like anti-IgE in combination with SIT using modified recombinant allergens would be desirable. This combination therapy may permit a safer use of SIT with modified allergens by reducing the risk of anaphylactic side effects due to therapy-induced IgE antibodies.

4.2. Tolerance and Th1-Inducing Adjuvants

Basically, class switch to IgE is induced when B cells interact with Th2 cells in the presence of IL4. These events can be influenced at different levels and a number of strategies have been developed that intend to suppress the Th2 driven class switch recombination. Blocking the IL-4 dependent actions of Th2 cells with antibodies against IL-4 represents another approach using antibodies as a therapeutic tool. From the immunological point of view, this approach will induce neither allergen specific nor long-lasting balancing of allergic responses, and moreover, IL-13 can replace several functional activities of IL-4. In general anti-IL4 and IL-5 treatment has been disappointing and contradictory [100]. An alternative was suggested by direct

blocking IL-4 and IL-13 synthesis. For this purpose mutant proteins acting as antagonists were developed [101].

Without any doubt, allergen-specific induction of tolerance would be the most elegant approach to protect from or to cure allergic diseases. In its optimal form, it would be free of any side effects and would lack the risk of strong Th1-biased approaches. Animal experiments indicated the potential of approaches such as local nasal immunotherapy [102, 103]. The role of IL-10 and TGF- β in the induction of anti-allergic tolerance was confirmed in animal experiments [104] and by clinical studies investigating the consequences of SIT on the immune status of patients [105]. Taken together, the data indicate that nasal or oral tolerance induction may be a potential approach for an alternative form of SIT. However, the results also indicate that the success of these attempts was restricted to the protective application with much less efficacy concerning a downregulation of already Th2-type responses.

With increasing insight into the mechanisms of Th1- and Th2-type immune responses, various possibilities to modulate Th2 immune response seem possible. The idea behind is, that Th1-biased stimuli could prevent the development of an allergic response. Bacille Calmette-Guerin (BCG) fulfils the criteria of a safe Th1 induction [106]. BCG in combination with allergen induced an anti-allergic effect in animal experiments. Another group of bacilli, the lactic bacteria (LAB) also proved to be anti-allergic candidates [107]. LPS represents a ubiquitous molecule of our environment and a number of important immunomodulatory processes have co-evolved with this molecule. With respect to allergy and asthma, the effect of LPS is still unclear. As an adjuvant co-administered with allergens partly improved the efficacy of SIT without triggering harmful side effects [108]. In parallel, stimulation of Th1 inducing cytokines like IFN- γ , IL-12 and IL-18 gave contradictory results in animal experiments [109, 110].

CONCLUSIONS

Recombinant allergens opened new perspectives for the diagnosis and treatment of allergic diseases. The concept of molecule-based instead of allergen extract-based diagnosis was developed and is already being implemented in the field. Information about the sensitization profile of individual patients forms the basis for the development of new and safer forms of immunotherapy based on the use of modified recombinant allergens. For this purpose, a variety of preparations are being developed for different allergies. Major goals are to increase safety by minimizing the risk of IgE-mediated side effects and to improve efficacy of SIT by counterbalancing the ongoing Th2-biased allergic response. Optimal vaccine preparations for SIT in the future should ideally include not only hypoallergenic molecules but also Th1-inducing adjuvants. The possibility of combining SIT based on modified recombinant allergens with alternative treatments such as anti-IgE also offers new perspectives.

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